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14. ABSTRACT Pheochromocytomas (PCC) are catecholamine-producing neuroendocrine tumors. Up to 30% give rise to metastases, for which there is no effective treatment. A deficiency in current treatment strategies is that they do not account for the fact that, in contrast to many other malignant tumors, metastatic PCCs usually grow very slowly and most of the cells are quiescent at any given time. Treatments that target replicating tumor cells have therefore been mostly unsuccessful. The need to improve treatment requires new strategies and valid models for pre-clinical testing. In this research period we: 1. Completed development and characterization of a bioluminescent mouse PCC cell line as a model, 2. Used the model in cytotoxicity assays to test two promising treatment strategies represented by different types of prototype drugs, 3. Validated the findings with human PCC cells in primary cultures. The first strategy tested was inhibition of topoisomerase 1 (TOP1) in conjunction with inhibition of DNA methylation. TOP1 inhibitors interfere with mechanisms that maintain DNA integrity during transcription in both quiescent and dividing cells, while DNA methylation inhibitors increase transcription of genes silenced by methylation, and are therefore mechanistically complementary. The second strategy tested a new type of drug, Gamitrinib, that accumulates in the mitochondria of tumor cells but not of normal cells, and attacks the chaperone protein TRAP-1. The rationale for testing Gamitrinib was that malignant PCC are often caused by mutations of the SDHB gene, which encodes a subunit of the mitochondrial enzyme succinate dehydrogenase. We found that Gamitrinib is highly toxic to both MPC cells and primary human PCC cells. It might therefore be both the most effective drug and the one most specifically targeting the defect in the majority of malignant PCC.					
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1. Introduction

Purpose and scope of the research effort.

Pheochromocytomas (PCC) are catecholamine producing neuroendocrine tumors that arise from chromaffin cells in the adrenal medulla or other parts of the peripheral sympathetic nervous system. Extra-adrenal PCCs are arbitrarily classified as paragangliomas (PGLs) (1). PCCs occur sporadically or in association with at least 10 hereditary diseases. Between 10% and 30% of PCCs give rise to metastases, for which there is currently no effective treatment. A major deficiency in current treatment strategies is that they do not account for the fact that, in contrast to many other types of malignant tumor, metastatic PCCs usually grow very slowly and most of the cells are quiescent at any given time. Mitotic counts and expression of cell cycle markers both in primary tumors and in their metastases are often very low. Treatments that target replicating tumor cells or tumor angiogenesis have therefore met with only limited success. Patients with metastases often die from complications of catecholamine hypersecretion, or from invasive and expansile tumor growth that occur over many years. The need to improve treatment of metastatic PCCs requires new strategies and a valid experimental model for pre-clinical testing of those strategies. Development of a model has itself been hampered by failure to establish any human PCC cell lines for cell culture or xenograft studies, despite many efforts to establish them and several initially promising reports.

This project aims both to develop treatment paradigms targeting quiescent and replicating tumor cells in PCC metastases using existing models generated in the PI's laboratory, and to develop new models. Specific Aim 1 uses a mouse pheochromocytoma cell line (MPC) *in vitro* as a model to test new approaches to enhance the effectiveness of chemotherapeutic agents by optimizing the combination and timing of their use. Aim 2 uses primary cultures of human PCC cells derived from individual patient's tumors with diverse genetic backgrounds to validate the findings in Aim 1, and Aim 3 tests the ability of drug-administration paradigms developed in specific aims 1 and 2 to kill disseminated PCC *in vivo* using MPC cells as a model in nude mice. Aim 4 tests novel approaches to develop cell lines of human PCC for cell culture and xenografts, thereby providing a foundation for future studies. Because PCC are rare tumors, an important aspect of the project is collaboration with other investigators in order to maximize access to human tumors and to new drugs.

2. Key Words

Pheochromocytoma, paraganglioma, cell culture, cell line, xenograft, nude mouse, chemotherapy, pre-clinical testing, camptothecin, 5-azacytidine, irinotecan, topoisomerase-1, bioluminescence, imaging

3. Overall Project Summary During the First Year (15 August 2011- 14 September 2012).

Section I. Progress on Individual Specific Aims

Specific Aim 1 *Development of drug testing paradigms using MPC cell cultures*

Summary: We completed apoptosis and cytotoxicity assays for prototype drugs tested on MPC cells in studies designed to test the efficacy of topoisomerase 1 (TOP1) inhibition in conjunction with inhibition of DNA methylation by 5-azacytidine (5-aza). TOP1 inhibitors are drugs that interfere with mechanisms that maintain DNA integrity during transcription in

both quiescent and dividing cells, while 5-aza is a DNA methylation inhibitor that alters transcription and is therefore mechanistically complementary. We also tested additional drugs representing potential alternative solutions and enhancements of the project.

Task 1. Cytotoxicity/survival/apoptosis assays were set up for use with MPC cells

- Four complementary procedures were set up; XTT colorimetric assay (measures cell survival by metabolism of a dye by viable cells), immunoblotting for cleaved poly-ADP-ribose polymerase (indicates apoptosis by showing a cleaved protein band generated by activated caspase 3), staining of cultures with 4'-6-diamidino-2-phenylindole (DAPI, 0.5 uM) (shows nuclear changes characteristic of apoptosis), ApoTox-Glo Triplex Assay (Promega Corporation, Madison WI, simultaneously assesses caspase activation, cytotoxicity and viability).

Task 2 . The effectiveness of TOP1 inhibition against MPC 4/30/PRR cell line was tested using camptothecin, a prototypical TOP1 inhibitor, to obtain proof of principle.

- Cells in monolayer cultures were tested with a range of camptothecin concentrations from 0.01 to 10 uM using XTT, PARP cleavage and DAPI stains as read-outs.
 - XTT Results showed approximately 20% survival in the presence of 1 uM camptothecin at 7 days and no survival at 7 days in the presence of 10 uM camptothecin.
 - PARP blots and DAPI stains confirmed the presence of ongoing apoptotic death over a 2-week period of testing.
 - These results were used as the basis for task 3 (See Figs 1-3 as described below)

Task 3 The effect of sub-toxic concentrations of 5-azacytidine (5-aza) in conjunction with camptothecin was tested as a possible strategy to reduce the required dosage or duration of treatment with TOP1 inhibitors.

- In order to test the interaction of camptothecin with 5-aza, cytotoxicity assays were performed with MPC 4/30PRR using 0, 0.5 or 1.0 μM camptothecin and a single low dose of 5-aza (1 μM) present during the first week and/or second week of a 2-week experiment. XTT, PARP cleavage and DAPI stains were used as read-outs.
 - All cultures treated with 5-aza plus camptothecin showed significantly reduced survival compared to those with camptothecin alone, with 5-aza optimally added during the first week. Further, those cultures from which 5-aza was removed after the first week were not significantly different from those with 5-aza continuously (Fig 1). PARP blots and DAPI stains confirmed increased apoptotic death with the 2-drug combination (Figs. 2 and 3).
 - These results suggest novel strategies for enhancing the efficacy and reducing the toxicity of TOP1 inhibitors by optimizing both the combination and timing of their use in conjunction with other drugs.

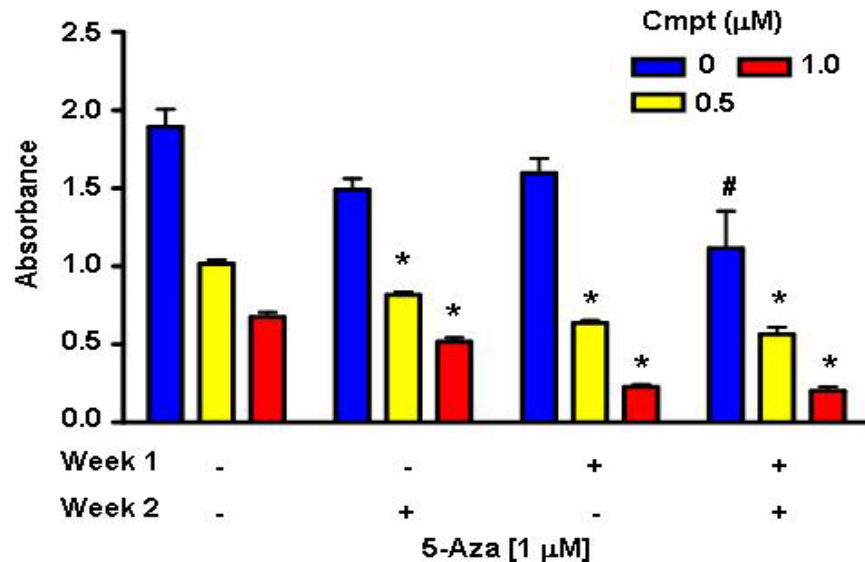


Fig 1. Cytotoxicity of camptothecin against MPC cells is increased by short-term concomitant exposure to a sub-toxic concentration of 5-azacytidine (1 μM). Captions under bars indicate whether 5-aza was present during the first week and/or second week of a 2-week experiment. In the absence of camptothecin, 5-aza alone causes a statistically significant decrease in survival only after 2 weeks of continuous treatment (#). Data are from a representative experiment that was repeated on 3 independent occasions. Absorbance is proportional to cell survival. Bars indicate mean \pm SEM of quadruplicate wells.

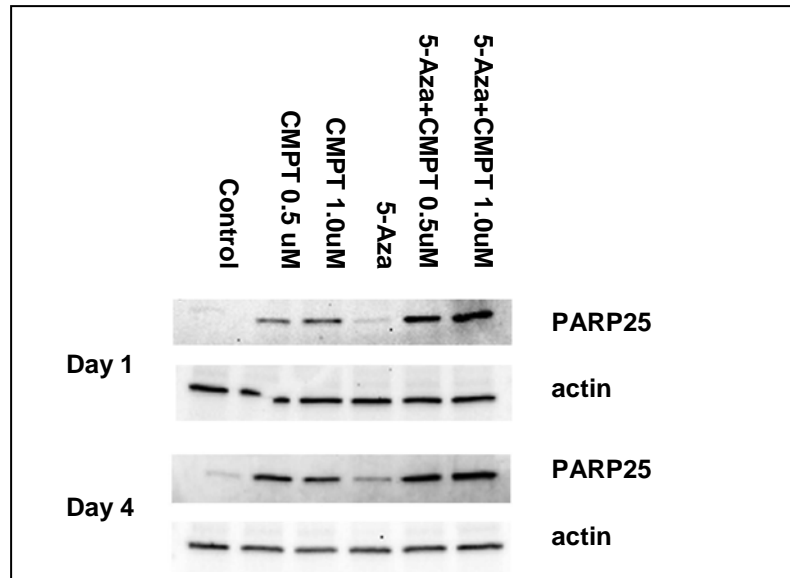


Fig. 2 Immunoblots showing the effects of camptothecin and 5-azacytidine on MPC cell apoptosis, which is indicated by the presence of a 25 kD fragment of PARP. A marked increase in intensity of the PARP25 band is seen at 24 hrs with the combination of camptothecin and 5-aza, with little effect of 5-aza alone. This pattern is still evident, but diminished, after 4 days.

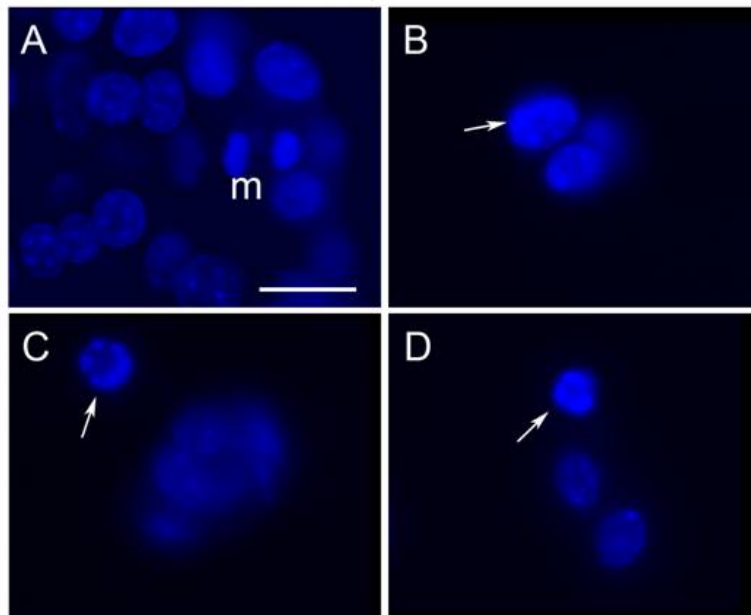
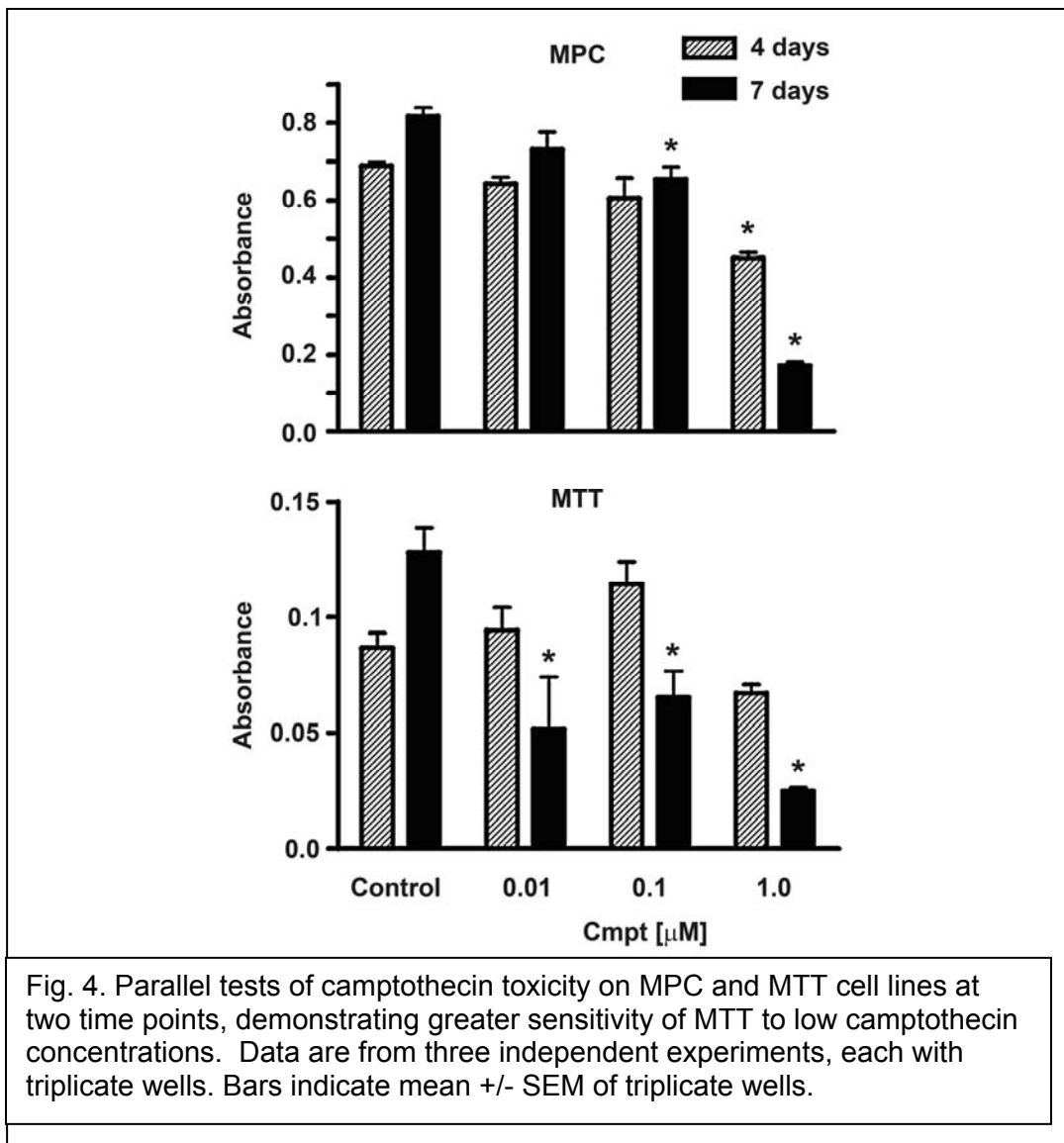


Fig.3 Representative fluorescence micrographs showing nuclear morphology of DAPI-stained MPC cultures. Panel A shows nuclei of cells maintained in control medium for 7 days. Nuclei are round to oval with finely stippled chromatin. One mitosis is evident (m). Panels B-D show typical apoptotic changes seen at day 7 in cultures with camptothecin or camptothecin + 5-aza. (B, early peripheral margination of chromatin; C, nuclear shrinkage and marked chromatin margination; d, nuclear fragmentation). In addition, B-D contain fewer cells, consistent with ongoing attrition. Bar =20 um. Original magnification 100 X.

Task 4. Because the amount of cell proliferation is an important consideration in the treatment of PCC, we compared the effects of camptothecin against MPC versus MTT, a rapidly growing cell line generated from MPC tumor tissue. MTT serves as a less differentiated counterpart to MPC, and, because of its rapid growth, representing the occasional human PCC that undergoes “explosive” growth after failure of chemotherapy.

- Comparisons of the MPC and MTT cell lines showed that both lines were sensitive to camptothecin, with MTT showing a lower threshold of response than MPC, but comparable cumulative toxicity (Fig. 4).



Task 5 Additional TOP1 inhibitors that are currently approved for clinical use were tested for comparison to camptothecin.

- Because native camptothecin is considered too toxic for clinical use, we tested three additional TOP1 inhibitors; topotecan, irinotecan and SN38, the active metabolite of irinotecan, against MPC cells for up to 2 weeks using the same methods as for Task 2. The concentration ranges tested (0.1-10 ng/mL for topotecan, 1-100 ng/mL for SN38) was chosen to match published *in vitro* tests of these drugs against other tumors
 - At its highest concentration, SN38 (100ng/mL=0.26 uM) was comparable in cytotoxicity to 1ug/mL camptothecin (=2.7uM), causing ~ 90% cell death, while topotecan at its highest concentration was less effective (<50% cell death). (Fig 5).
 - This result formed the basis for proceeding with irinotecan as the camptothecin analog to be tested *in vivo* during the next funding period.

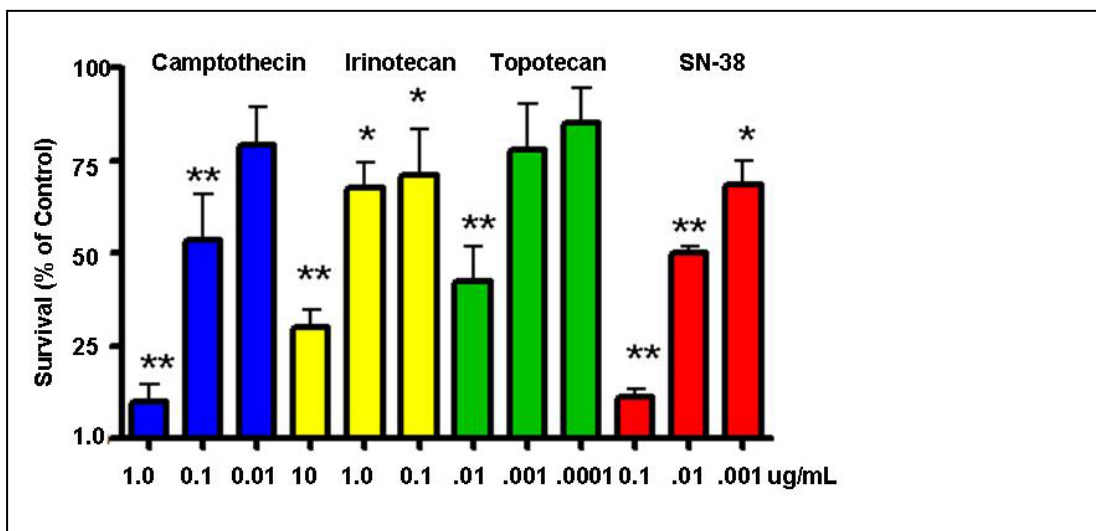


Fig. 5 Concurrent tests of camptothecin versus clinically utilized analogs on MPC cells in monolayer cultures at one week. Comparable sensitivity to camptothecin and SN-38 is seen at the indicated concentrations. Data are from three independent experiments, each with triplicate wells. Bars indicate mean \pm SEM. (**, $p < .01$; *, $p < .05$)

Task 6 As a prelude to *in vivo* testing, we tested to determine whether 3-dimensional growth affects drug sensitivity. This was done because tumors *in vivo* grow in 3 dimensions.

- Using the same methods as in Task 5, we tested the efficacy of camptothecin and its analogs against MPC cells grown as spherical clusters in suspension.

- Both camptothecin and SN-38 were effective against MPC cells in 3-dimensional as well as monolayer cultures. However, under otherwise identical test conditions, the same drug concentrations were less effective against 3-dimensional clusters than monolayer cultures (Fig. 6).

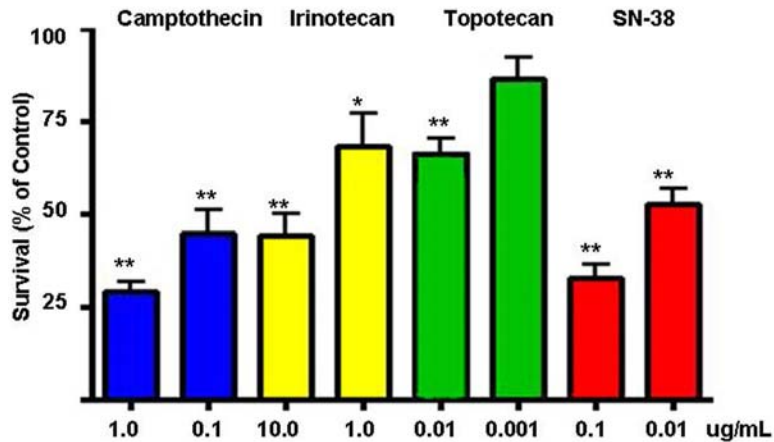


Fig. 6 Concurrent tests of camptothecin versus clinically utilized analogs on MPC cells in 3-dimensional cultures at one week. Comparable sensitivity to camptothecin and SN-38 is seen at the indicated concentrations. However, for both drugs, fewer cells are killed in 3-dimensional cultures than in monolayer cultures during the same time frame (see Fig. 5). Data are from three independent experiments, each with triplicate wells. Bars indicate mean \pm SEM. (**, $p < .01$; *, $p < .05$)

Task 7. Two alternative drugs were tested as possible alternatives to TOP1 inhibitors

- Using the XTT assay as a screen as in Task 2, we tested a completely new type of drug known as Gamitrinib, developed by our collaborator, Dario Altieri at the Wistar Institute. Gamitrinib accumulates in the mitochondria of tumor cells but not of normal cells, and attacks a mitochondrial chaperone protein called TRAP-1. Cytotoxic effects were measured over a 2 week period.
 - At a concentration of 10 uM, Gamitrinib caused 60% MPC cell death at 1 week and > 90% death at 2 weeks, putting it on a par with camptothecin as a potentially useful drug. (Fig. 7)

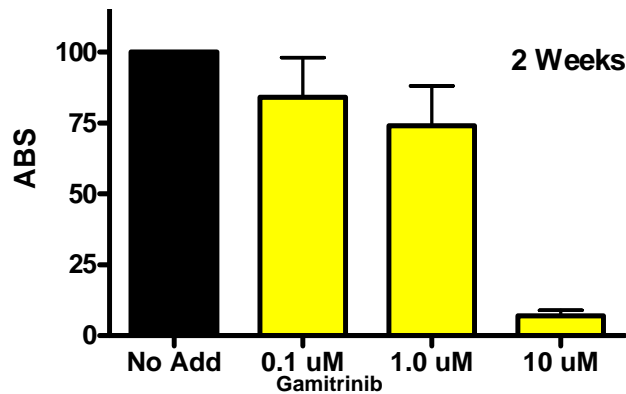


Fig. 7 Toxicity of Gamitrinib to MPC 4/30/PRR, measured by XTT colorimetric assay. Data are from 3 independent experiments with triplicate wells. An unusual aspect of the gamitrinib response is an abrupt dose-response curve with a threshold between 1 and 10 uM

- In collaboration with our NIH collaborators, we tested a drug called Torin-1, representing a new class of agents directed against mTOR, the mammalian target of rapamycin, a master regulator of cell proliferation and survival. Subsets of PCC show elevated mTOR signaling. Torin was tested against MTT, an aggressive, rapidly growing derivative of our MPC cells, using the MTT colorimetric assay.
 - With this aggressive cell line, Torin-1 was able to significantly inhibit cellular proliferation in a dose-dependent manner over a range of concentrations (1nM to 1 μ M) with an IC₅₀ of 207 nM and 80% cell death within 48 hours. However, it was much less effective than either camptothecin or Gamitrinib in primary human PCC cultures (see Specific Aim 2 below). and is therefore likely to have limited usefulness

Specific Aim 2 Validation of drug testing paradigms in primary human cell cultures

Summary: We tested human tumors in primary cultures with the most promising drugs identified in Specific Aim 1

Task 1. Camptothecin was tested to confirm its cytotoxicity against primary cultures of representative human pheochromocytomas.

- Long-term killing of cells from 7 human PCC/PGL with diverse genetic backgrounds was tested in primary cultures. This test series included 3 tumors

with SDHB mutations, the genotype most prone to metastasize. The cultures were maintained with 0 (control) (n=7), 1uM (n=7) or 10 uM (n=3) camptothecin for 2 wks, then fixed and stained for tyrosine hydroxylase to discriminate the tumor cells from other cell types. Counts of surviving cells were derived by counting all stained cells defined by a randomly placed square coverslip in a 35 mm culture dish. (Fig 8, Table 1)

- Mean tumor cell survival at week 2 was 47 % with 1 uM and 25 % with 10 uM camptothecin (Fig 8, Table 1). All tumors responded, but responses for individual tumors ranged. The lower sensitivity of human PCC vs MPC to camptothecin probably reflects the fact that MPC cells proliferate in culture while human PCC do not. It emphasizes the importance of comparing the human and mouse models and the need to develop effective multidrug strategies for the human tumors.

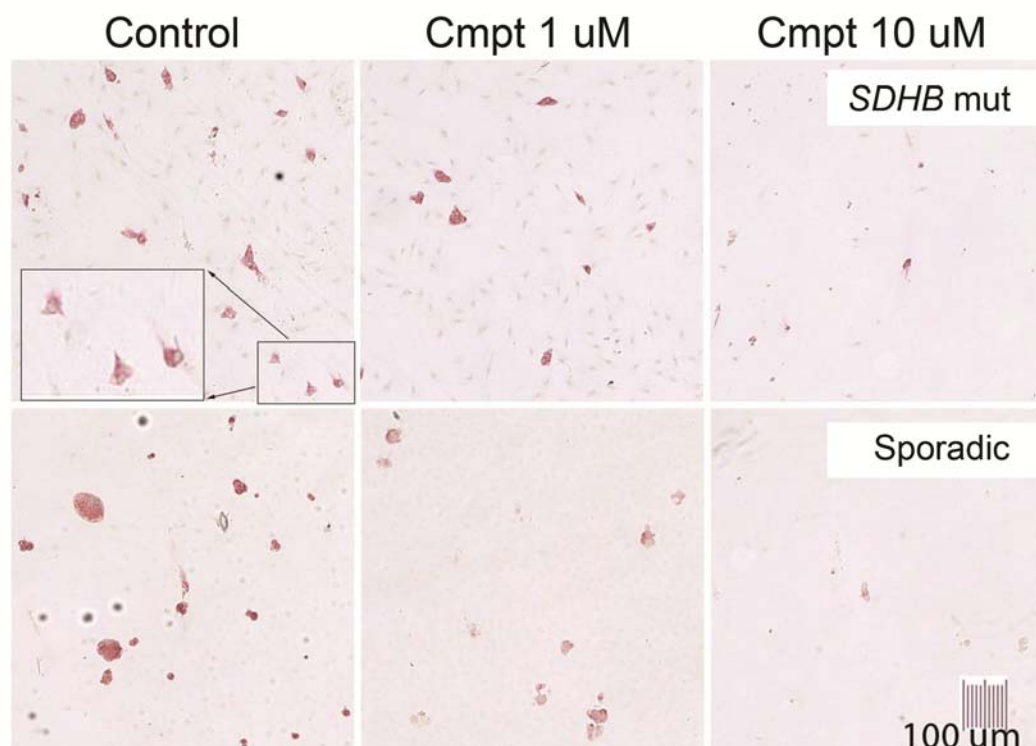


Fig. 8 A. Long-term killing of cells from representative human PCC/PGL s by camptothecin (Cmpt) in primary cultures maintained with 0 (control), 1 or 10 uM Cmpt for 2 wks, then fixed and stained for TH (red cytoplasm) to discriminate the tumor cells from other cell types. At 10 uM, camptothecin eliminated almost all background cells (faintly visible as hematoxylin-counterstained blue nuclei in top row control and 1 uM panels). NOTE: the tumor in the bottom row was identified as *RET*-mutated in an earlier version of this progress report. The genotype data were subsequently corrected.

Table 1. Survival of tumor cells from 7 individual PCCs / PGLs cultured in the presence of 1 uM or 10 uM camptothecin compared to control medium.

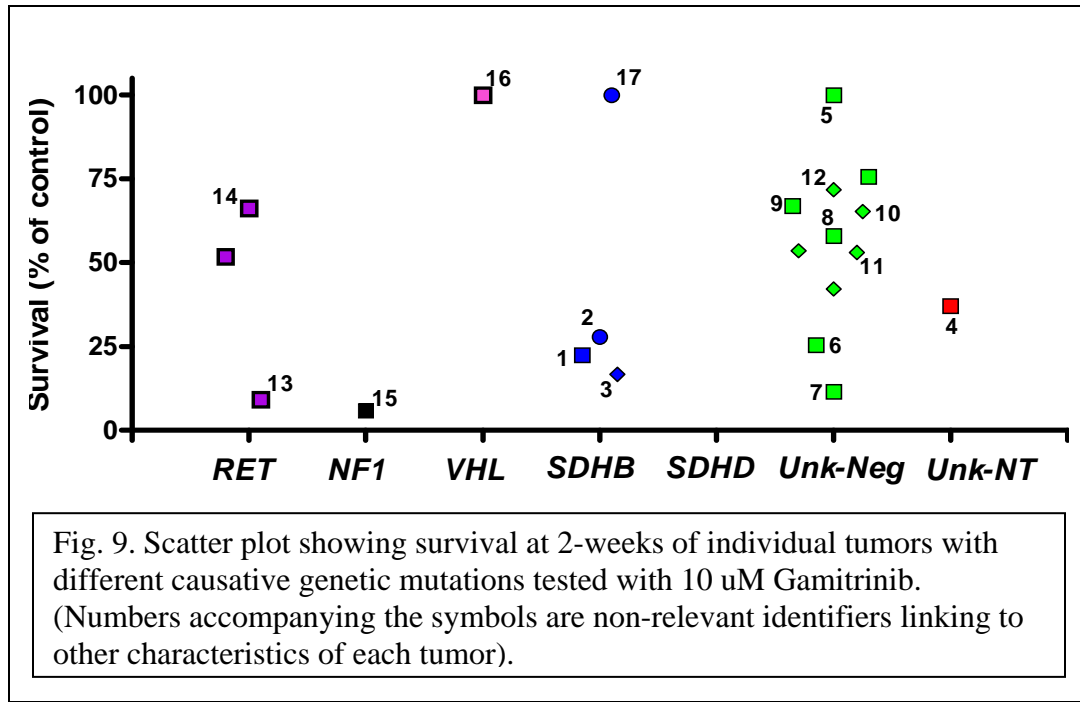
Tumor	Genotype	Surviving Cells/Dish (% of Control)*	
		1.0 uM Cmpt	10 uM Cmpt
1 (V49) PCC	VHL	39.1	18.6
2 (S116) PCC	Sporadic-Neg	63.3	29.1
3 (M26A) PCC	Sporadic -Neg	18.6	4.7
4 (TS12-2159) PCC	Unknown NT	45.7	
5 (S111B) PGL	SDHB	33.1	2.2
6 (S189) PGL	SDHB	71.7	
7** (S123) PGL	SDHB	24.0**	10.1

*Counts were derived by counting all stained cells defined by a randomly placed square coverslip in a 35 mm culture dish. All counts were done at 2 weeks except for tumor 7 (**), which was counted at 1 week because of extensive cell death caused by particular sensitivity to camptothecin. Only 5 of the tumors were tested at 10 uM because that concentration eliminated almost all background cells and was therefore considered to exert too much bystander toxicity.

Task 2. Gamitrinib was tested as an alternative drug

- We completed testing a series of 21 PCC/PGL representing different genotypes and locations for response to Gamitrinib. This study was begun prior to DoD funding but was continued as the potential usefulness of the drug became clear. Gamitrinib was tested at a concentration of 10 uM based on the results of Specific Aim 1, using the same methods as for Task 1
 - Individual tumors, shown as symbols in the scatter plot below showing survival at 2-weeks (Fig 9) were variably sensitive.
 - Importantly, 3 of 4 tumors caused by mutations of the *SDHB* gene were highly sensitive. PCC/PGL with *SDHB* mutations are the most likely to be malignant. *SDHB* encodes a subunit of the mitochondrial enzyme succinate dehydrogenase, and therefore show impaired electron transport and pseudo-hypoxic signaling. Because it targets mitochondria in tumors that are driven by abnormal mitochondrial function, Gamitrinib may be both the most effective drug and the one most specifically targeting the majority of malignant PCC. Because Gamitrinib was highly effective against human PCC with relatively little bystander toxicity, we plan to

begin our *in vivo* testing with Gamitrinib rather than a TOP-1 inhibitor in the next funding period.



Specific Aim 3 In vivo testing of drug-administration paradigms developed in specific aims 1 and 2 using disseminated MPC cells as a model

Summary: We modified the mouse pheochromocytoma cell line used in specific aim 1 to create a novel bioluminescence model for *in vivo* testing .

Task 1 As our principal model for *in vivo* testing, we generated a cell line designated MPC 4/30/PRR GL-9 (abbreviated to MPC GL-9) that expresses green fluorescent protein (GFP) and firefly luciferase. The luciferase emits light in mice injected with the substrate luciferin, permitting quantitation of tumor growth or regression at repeated intervals using a luminometer designed for small animal imaging, while the GFP permits the cells to be sorted in a cell sorter in order to maintain a stable phenotype or recover pure tumor cells from *in vivo* deposits

- MPC GL-9 was derived from MPC 4/30PRR. by transducing the cells with a pre-packaged lentiviral construct (GreenFire1, SBI Systems) containing both the GFP and luciferase genes under control of the CMV promoter. Infection was performed according to the manufacturer's protocol. MPC GL-9, which stably expresses high levels of luciferase, was cloned from a single transduced cell identified by its GFP fluorescence.. Clones were initially isolated with glass cloning cylinders and then with a fluorescence activated cell

sorter (Fig. 10) Aside from expression of its two marker proteins, MPC G-L9 is similar to its parent tumor.

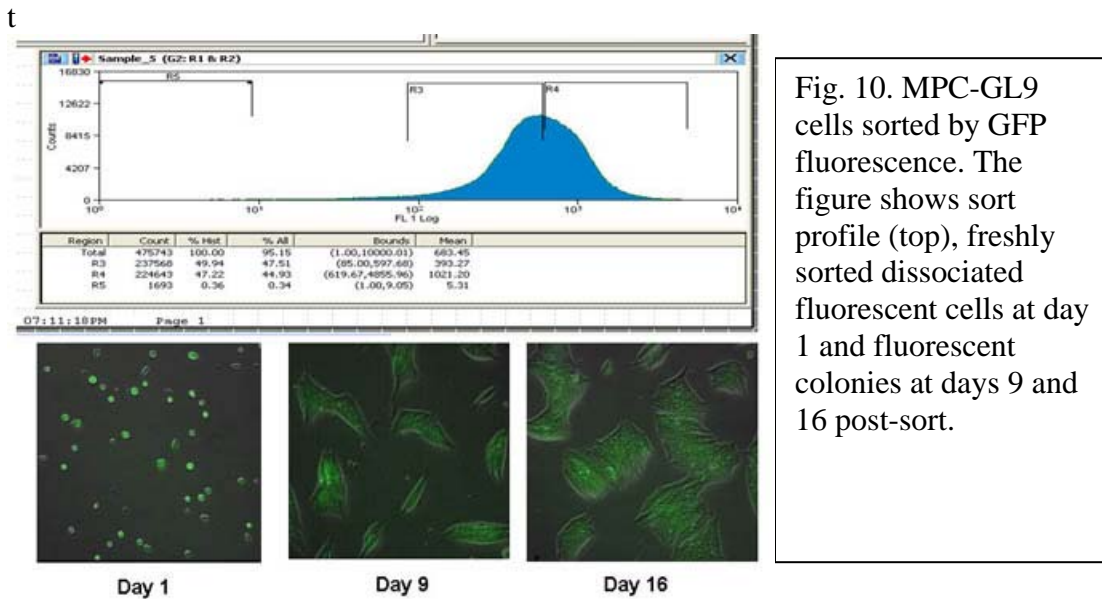


Fig. 10. MPC-GL9 cells sorted by GFP fluorescence. The figure shows sort profile (top), freshly sorted dissociated fluorescent cells at day 1 and fluorescent colonies at days 9 and 16 post-sort.

Task 2 The ability of MPC GL-9 cells to emit detectable light was tested *in vivo* in order to develop protocols *for in vivo* drug testing.

- 500,000 MPC-GL9 cells (equivalent to a moderately dense small culture dish) were injected into immunodeficient mice by tail vein or subcutaneous injection. The mice were anesthetized and given an intraperitoneal injection of luciferin. After 5 min the mice were imaged in a Xenogen IVIS 200 biophotonic imager at the Tufts Small Animal Imaging Facility.
 - Luminescent cells were immediately detectable, distributed between both lungs after tail vein injection (Fig. 11) or in subcutaneous sites. This model is therefore ready for all of our *in vivo* imaging studies.

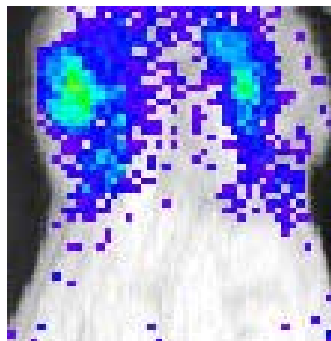


Fig. 11 *In vivo* luminescence of a NOD-SCID mouse imaged immediately after tail vein injection of 500,000 MPC-GL9 cells.

Task 3 We collaborated with colleagues at the NIH on a second luminescent cell line complementary to MPC-GL9

- Starting with MPC 4/30PRR cells from our laboratory, our NIH collaborators generated a luminescent cell line known as MTT-Luc. MPC 4/30PRR cells were first injected into tail veins. A rapidly growing cell line designated MTT (for Mouse Tumor Tissue) was cultured from resulting disseminated tumor deposits. Luciferase driven by the CMV promoter was then introduced using a retrovirus vector to create MTT-Luc.
 - MTT-Luc and its non-luminescent counterpart MTT serve as a rapidly growing counterpart to the better differentiated MPC GL-9, representing the occasional human PCC that undergoes “explosive” growth after failure of chemotherapy.
 - A paper describing MTT-Luc has been published (Giubellino A, Woldemichael GM, Sourbier C, Lizak MJ, Powers JF, Tischler AS & Pacak K 2012 Characterization of two mouse models of metastatic pheochromocytoma using bioluminescence imaging. *Cancer Lett* **316** 46-52) PMID:22154086 .

Specific Aim 4 Novel approaches to develop cell lines of human PCC for cell culture and xenografts

Summary: For this aim we pursued two parallel tracks: Attempts to directly establish cell lines from primary human PCC cell cultures using novel culture conditions, and attempts to establish propagatable xenografts by direct introduction of human PCC cells into favored metastatic sites.

Task 1 Tumor cell proliferation was tested under hypoxic compared to routine normoxic cell culture conditions

- We studied 23 PCC/PGL representing different genotypes. Hypoxic culturing was performed in modular incubator chambers (from Billups-Rothenberg Inc) with pre-mixed atmospheres containing 1% -4% O₂. Proliferation was assessed by labeling with bromodeoxyuridine (BrdU) as a marker for DNA replication. BrdU was present in the culture medium for 6 days or one week prior to fixation. Double immunohistochemical staining was performed for BrdU and tyrosine hydroxylase to discriminate tumor cells from proliferating fibroblasts and other cell types in primary cultures. Some cultures were studied at two or more time points (Fig. 13). For some tumors, preliminary studies were also performed testing hypoxia/normoxia in several culture media and in media with growth factor or other supplements as shown in Table 2.
 - There was no BrdU labeling of tumor cells in either normoxic or hypoxic cultures under any conditions, while labeling of irrelevant cell types was readily detected (Fig 13, Table 2). Because the hypoxic culture approach was unsuccessful, we suspended this work in order to evaluate additional options (see Areas of Concern section to follow) while focusing on more

promising xenograft approaches. Frozen cells from most of the tumors are available for future cell culture experiments.

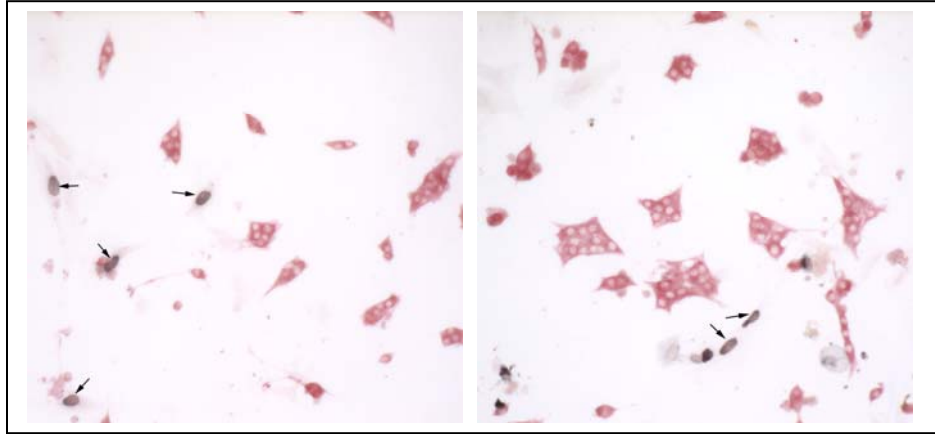


Fig.13. Representative cultures of cells from a human pheochromocytoma in normoxic (left, 20% O₂) *versus* hypoxic (right, 4% O₂) cultures. BrdU was added to the medium immediately on plating. After 7 days, cultures were fixed and double stained for BrdU (black nuclei) and tyrosine hydroxylase (red cytoplasm). BrdU labeling of fibroblasts is seen under both conditions (arrows), while pheochromocytoma cells show no BrdU incorporation. Numerous pheochromocytoma cells, which are smaller than fibroblasts, are seen in clusters. Clear circles within the red clusters are unlabeled nuclei.

Table 2. Summary of Human Tumors Tested for BrdU Incorporation in Hypoxic vs. Normoxic Conditions*									
	Sex	Age	Genotype	Site	%O ₂ RPMI	%O ₂ RPMI-HG	%O ₂ RPMI-CS	%O ₂ NSC	Hypoxia +adds
1	F	23	sporadic-Neg	R adrenal	2%-6 d				
2	F	26	sporadic-Neg	L adrenal	3%-18 d			3%-18 d	
3	F		sporadic-Neg		3%-18 d			3%-18 d	
4	M	18	sporadic-Neg	R adrenal	3%-18 d			3%-18 d	
5	M	31	VHL	R adrenal	3%-18 d			3%-18 d	
6	M	41	sporadic-Neg	R adrenal	3%-18 d			3%-18 d	
7	M	16.4	SDHB	Bladder	3%-1, 4 wk				
8	M	39	SDHB	Met lung	4%-1,3 wk		4%-1wk	4%-1 wk	EGF, FGF 1 wk, FA1, FA2,

									VPA 1,3 wk
9	M	54	?SDHB	Met	4%- 1 wk			4%- 1 wk	EGF, FGF, EGF+FGF, NSC suppl- 1wk
10	M	48	SDHB	met-liver	4%- 1 wk,				
11	M	45	SDHB	Retroperi toneal	4%- 1,8 wk				
12	M	?	sporadic- Neg	R adrenal	4%- 1wk				
13	F	29	sporadic- Neg	Infra aortic	4%- 1 wk				IGF II- 7 d
14	F			R perirenal	4%- 1 wk				IGF II- 7 d
15	M	44	sporadic- Neg	R adrenal	4%- 1 wk				IGF II- 7 d
16	M	33	SDHD	L carotid	4%- 1,2,10 wk				
17	F	66	Sporadic ? Not tested	R adrenal	1% - 1,3, 8 wk	1% - 1,3, 8 wk			
18	M	33	sporadic- neg	Heart	1% -1,3, 8 wk	1% - 1,3, 8 wk			
19	M	27	SDHB	Para- spinal	1%- 1 wk	1%- 1,3 wk			
20	F	50	sporadic- neg	R thorax	1%- 1, 2, 8 wk	1%- 1, 2, 8 wk			
21	F	?	RET/ MEN2A			4% and 1%- 1, 2, 6 wk			
22	M	34	SDHC	Heart	4%- 1 wk, 60 d				50% FBS- 14, 60 d
23	M	74	Unk		4%- 1 wk				
	M	58	Normal Adrenal medulla		3%-1 wk			Float vs. Att- 1 wk	EGF, FGF, NSC suppl

SUMMARY

Total # of Tumors	23
male	15
female	8

Sporadic (Neg genetic tests)	10
Indefinite	5
Definite SDHB	4
Definite SDHC	1
Definite SDHD	1
Definite VHL	1
Definite RET MEN2A	1

*TABLE LEGEND. All tumors were tested for BrdU incorporation in normoxic (20% O₂) vs hypoxic cultures at the indicated O₂ concentrations and time points. The routine medium for testing all tumors was RPMI 1640 with 15% fetal bovine serum ("RPMI"). For some tumors, additional parallel testing was performed in High Glucose RPMI 1640 with 15% FBS ("RPMI-HG"), RPMI with 15% calf serum ("RPMI-CS"), or synthetic neural stem cell medium ("NSC"). Some tumors were also tested in RPMI 1640 with 15% FBS plus the indicated supplements (EGF, epidermal growth factor; FGF, basic fibroblast growth factor; FA1 & FA2, fatty acid supplements 1 & 2 from Sigma Chemical Co.; IGF II, insulin-like growth factor 2; NSC, neural stem cell supplement, from Neural Stem Cell Technologies Inc.).

Task 2 Approval was obtained from Tufts IACUC for different methods of xenografting human PCC.

Task 3 Pilot studies were performed testing surgical implantation human PCC into the liver.

- A small abdominal incision was made in anesthetized mice. The right lobe of the liver was protruded through the incision. A small tumor fragment was implanted through an incision in the liver capsule. The liver capsule incision was rapidly sealed with Vetbond adhesive, the liver lobe was returned to its normal location and the abdominal incision was closed with surgical staples.
 - This method permitted tumor fragments to be retained in the liver and subsequently studied histologically.

Task 4 Pilot studies were performed for intrahepatic injection of dissociated human PCC cells into the liver

- Injection of dissociated tumor cells into the right lobe of the liver was tested both with the liver *in situ* under visualization by small animal ultrasound and with surgically exposed liver as in Task 3. Cells were injected in 10 uL of tissue culture medium.
 - This method was unsuccessful because intrahepatic pressure forced most of the cells out of the liver despite the low injection volume. This method was consequently abandoned.

Task 5 The bioluminescence methodology from specific aim 3 was adapted to develop a method for tracking the survival of human tumor xenografts

- Transduction of dissociated human PCC cells was first attempted with a lentivirus-luciferase construct using the same method that was successful for MPC cells in specific aim 3. Viral multiplicity of infection (MOI) of 5-50 was tested in medium containing 0-8ug polybrene/mL.
 - Under all conditions infectivity was extremely low ($\ll 1/1000$ cells).
- Because of the unsatisfactory infectivity with lentivirus, we next tested an adenoviral luciferase vector (AD-CMV-luciferase, Vector Biolabs).
 - At MOI 10, AD-CMV-luciferase was found to infect 100% of PCC cells.
- To obtain proof of principle, AD-CMV-luciferase was used to test survival of representative intrahepatic xenografts. Small tissue fragments were incubated overnight with AD-CMV-luciferase in complete culture medium. The virus was then washed out and cultures maintained for 1 week with 2 intervening medium changes. Tissue fragments were then implanted into the liver as in Task 2. At intervals the mice were anesthetized and given an intraperitoneal injection of

luciferin. After 5 min they were imaged in a Xenogen IVIS 200 biophotonic imager at the Tufts Small Animal Imaging Facility

- Successfully grafted tissue shows a single brightly luminescent signal (Fig 14).
- Graft survival will be monitored in a series of tumors in the next funding period.

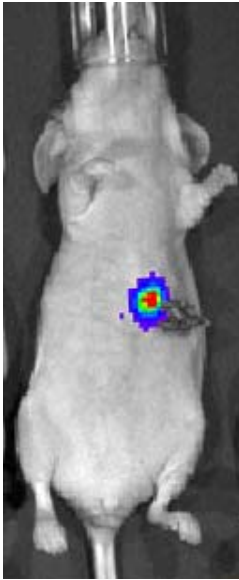


Fig.14. Bioluminescence image of an anesthetized mouse with an intrahepatic graft of a human PCC tissue fragment expressing adenovirus-luciferase, 4 days post-grafting. The graft was implanted in the right lobe of the liver, which is shifted somewhat to the left in the photograph. This graft failed to vascularize and did not survive an additional week.

Section II - Problem Areas

1. We have not yet been able to develop a high throughput method for cytotoxicity testing of primary human PCC cultures. We attempted to develop a cell-based ELISA for tyrosine hydroxylase as a specific indicator of PCC cell survival but could not achieve sufficient sensitivity. We have also unsuccessfully attempted to use variations of a fluorometric microculture cytotoxicity assay (Nat Protoc. 3: 1364-1369, 2008). We will next attempt to develop a bioluminescence –based assay as discussed in Goal 3 section IV.

2. The failure of hypoxic culture conditions alone to stimulate proliferation of human PCC cells in Specific Aim 4 caused us to suspend work on that part of the project while considering additional approaches. Our next step will be to perform pilot studies testing hypoxia in conjunction with small molecules that we reported many years ago to be mitogenic for normal rat chromaffin cells (Tischler AS, Riseberg JC, Cherington V. Multiple mitogenic signalling pathways in chromaffin cells: a model for cell cycle regulation in the nervous system. *Neurosci Lett* 168: 181-184, 1994 PMID 7518060). The testing of additional approaches is discussed in Goal 5 section IV.

3. We have used an adenovirus construct to develop a novel method for tracking survival of primary tumor xenografts. However, unlike lentivirus, adenovirus constructs are not integrated into the genome and are therefore progressively lost from dividing cell populations. Because human PCC cells replicate slowly or not at all, adenovirus-luciferase serves as at least a short term indicator of their location and survival. We are testing methods to improve the ability of lentivirus to transduce human PCC cells tissue in order to track grafts that might proliferate (Goal 4).

4. On histologic examination, intrahepatic xenografts tested thus far showed failure to vascularize. We are currently working to compare intrahepatic versus tail vein and subcutaneous injection as the optimal graft site and to finalize the choice of mouse strain before starting a large series of xenografts (Goal 4).

Section III - Description of work to be performed during the next reporting period.

Goal 1. Marshall 3 papers thus far written, but not published, through the review process to publication.

Task 1. Respond to critiques, perform additional experiments as required, resubmit papers.

Goal 2. Follow up promising results thus far obtained in specific aims 1 and 2

Task 1. Test toxicity of Gamitrinib against disseminated MPC GL-9 cells in nude mice ~ 2 months).

- 1a. Propagate sufficient cells to inject 20 mice, 10^6 cells/mouse (3 weeks)
- 1b. Wait for discrete tumor masses to form. (1 month)
- 1c. One-month treatment protocol with luminescence imaging (4 weeks).
- 1d. Harvest tumor tissue from control and treated animals at termination of experiment for histologic sections and RNA extraction.

Task 2. Cell culture testing of topoisomerase inhibitors in conjunction with histone deacetylase inhibitors using MPC cells as described in project narrative and approved SOW (ongoing, 12 months).

Task 3. Cell culture testing of topoisomerase inhibitors in conjunction with alternate drugs including lithium and caffeine using MPC cells as described in project narrative and approved SOW (ongoing, 12 months).

Goal 3. Attempt to develop high throughput bioluminescence –based cytotoxicity assay for human PCC in cell cultures (Problem Area 1)

Task 1. Perform dilution curve with luminescent MPC GL-9 cells to determine minimal number of cells detectable, linear response range, and response to a representative drug already tested (3 weeks)

Task 2.- If sensitivity appears adequate judging from Task 1, test above assay on 3 representative human PCC using adenovirus-luciferase construct to make primary cultures luminescent (time frame dependent on procurement of new tumors)

Task 3.- If sensitivity for human PCC is judged adequate based on Task 2, implement the assay for subsequent testing of human tumors. If not, continue to use the current immunohistochemistry-based assay.

Goal 4. Finalize choice for route of administration of human PCC cells to mice and choice of mouse strain (Specific Aim 4 in progress report).

Task 1. Compare survival of luminescent human PCC introduced by tail vein vs intrahepatic injection in 3 mouse strains (nude, SCID, NOD-SCID, 10 mice each).

Task 2. Attempt to develop protocol for using lentivirus for transduction of human PCC with luciferase by varying multiplicity of infection and testing polybrene vs new commercial reagents to optimize efficiency.

Task 3. Based on Tasks 1 and 2, choose one optimal protocol for continuation of Specific Aim 4 through month 36 as described in the Project Narrative and approved SOW.

Goal 5. Attempt to address lack of human PCC cell proliferative response to hypoxic culture conditions (Problem Area 2, Specific Aim 4A)

Task 1. Test 5 PCC representing different genotypes for proliferation under hypoxic vs normoxic conditions in the presence and absence of small molecules previously shown to be mitogenic for rat chromaffin cells (i.e. forskolin, an activator of adenylate cyclase) and phorbol acetate (an activator of protein kinase). (time frame dependent on procurement of new tumors). If there is no response to these modifications of the protocol, we will have to consider a completely different approach to Specific Aim 4A.

5. Key Research Accomplishments During the First Year (15 August 2011- 14 September 2012).

- Showed that camptothecin, a prototypical topoisomerase-1 (Topo-1) inhibitor, is cytotoxic to non-replicating human pheochromocytoma cells in primary cultures. This provides proof of principle for using Topo-1 inhibitors to

target slowly growing but hormonally active, therefore potentially lethal, pheochromocytoma metastases.

- Showed that SN-38, the active metabolite of irinotecan, a Topo-1 inhibitor currently in clinical use, is cytotoxic against cell cultures of a mouse pheochromocytoma cell line that serves as a model for pre-clinical drug testing.
- Showed that the efficacy of SN-38 in cell cultures is increased by brief concurrent exposure to a non-toxic concentration of 5-azacytidine, a DNA methyltransferase inhibitor. This provides proof of principle for designing *in vivo* strategies using Topo-1 inhibitors in conjunction with a mechanistically complementary drug.
- Showed that Gamitrinib, a totally new type of drug with potentially less bystander toxicity than Topo-1 inhibitors, is as effective as camptothecin against both human and mouse pheochromocytoma in cell cultures. This provides a rationale for next studying both irinotecan and Gamitrinib in mouse xenograft studies.
- Developed a bioluminescent mouse pheochromocytoma cell line that will facilitate all *in vivo* pre-clinical drug testing in mouse xenograft studies.
- Developed a novel method for transiently tagging primary human pheochromocytoma cells with a bioluminescence marker using an adenoviral vector. This will be an important tool for monitoring xenograft survival and will thereby facilitate efforts to establish human pheochromocytoma cell lines, which are much needed and currently do not exist.
- Showed that hypoxia alone is not sufficient to trigger proliferation of human pheochromocytoma cells in culture. Although this finding is disappointing in terms of efforts to establish human cell lines, the data are mechanistically important because hypoxic signaling is believed to play important roles in the development of pheochromocytomas, including those with *SDHB* mutations

5. Conclusion

We have made progress on all of the tasks in the approved SOW and Project Narrative. We are ahead of schedule for Specific Aims 1 and 2, having completed apoptosis and cytotoxicity assays for prototype drugs tested on MPC cells in studies designed to test the efficacy of topoisomerase 1 inhibition in conjunction with inhibition of DNA methylation. We also tested additional drugs representing potential alternative solutions and enhancements of the project. Three papers directly pertaining to Specific Aims 1 and 2 are either already submitted for publication or about to be submitted. One paper pertaining to specific Aim 3 has already been published. The most challenging component of the project concerns attempts to establish human PCC cell lines, which currently do not exist despite attempts in many laboratories to establish them over a period of at least 35 years. We have introduced a promising approach to human PCC xenografting that permits the viability and distribution of grafted cells to be assessed in live mice by bioluminescence imaging. That approach will be refined and pursued in the

next performance period. Our novel attempts to directly establish cell lines from primary cultures under hypoxic conditions have not worked, and modifications of the protocol will therefore be tested in the next performance period.

6. Publications, Abstracts and Presentations

Publication during the first performance period:

Giubellino A, Woldemichael GM, Sourbier C, Lizak MJ, Powers JF, Tischler AS & Pacak K 2012 Characterization of two mouse models of metastatic pheochromocytoma using bioluminescence imaging. *Cancer Lett* **316** 46-52. PMID:22154086

Note: The acknowledgement of this DoD grant in the above paper inadvertently used the incorrect number 10665589, which was the number on our Grants.gov submission receipt for the proposal, dated 4 August 2010.

7. Inventions, Patents and Licenses None

8. Reportable Outcomes None

9. Other Achievements Human pheochromocytoma tissues collected with the support of this grant will be contributed to The Cancer Genome Atlas project. Pheochromocytomas are the first rare tumors to be studied by TCGA. Because of their rarity, our tumors will constitute a substantial percentage of the total number available and will make a substantial contribution to understanding of this disease.

10. References New citations are included in text

11. Appendices None